PAUF is overexpressed in pancreatic cancer stem cells and confers chemoresistance.

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PAUF is overexpressed in pancreatic cancer stem cells and confers chemoresistance.

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<TABLE OF CONTENTS>

ABSTRACT ····· 1

I. INTRODUCTION ····· 3

II. MATERIALS AND METHODS

1. Clinical samples and cell lines ····· 5
2. Sphere culture ····· 5
3. Semi-quantitative PCR (reverse transcription PCR) ····· 6
4. Establishment of stable PAUF knockdown cell line using shRNA ····· 8
5. Transient transfection of small interfering RNA (siRNA) targeted for PAUF. ····· 8
6. MTT assay ····· 9
7. Flow cytometry and cell sorting ····· 9
8. Protein extraction and western blot ····· 10
9. Immunohistochemistry ····· 11
10. Soft agar assay ····· 11
11. Statistical analysis ····· 12

III. RESULTS

1. Expression of PAUF protein in human pancreatic cancer ····· 13
2. Pancreatic cancer spheres cultured from human pancreatic cancer cell lines ····· 14
3. PAUF overexpression in pancreatic CSCs ····· 15
4. PAUF knockdown effect on pancreatic CSCs ····· 16
5. Effects of PAUF on chemoresistance of pancreatic cancer cell lines ····· 20

IV. DISCUSSION ····· 23
V. CONCLUSION ····· 26

REFERENCES ····· 27
ABSTRACT(IN KOREAN) ····· 32

PUBLICATION LIST ····· 34
ACRONYMS LIST ····· 35

LIST OF FIGURES

Figure 1. Validation of PAUF expression in pancreatic tissues …… 13

Figure 2. Expression of pluripotent stemness genes in pancreatic cancer spheres formed from CFPAC-1 cells …… 14

Figure 3. PAUF overexpression in pancreatic cancer stem cells …… 15

Figure 4. Effect of PAUF knockdown on sphere- and colony-forming ability…… 17

Figure 5. Differential gene expression in PAUF knockdown CFPAC-1 cells…… 18

Figure 6. PAUF knockdown effects on the expression of pancreatic cancer stem cell markers (CD24/CD44/ESA) in CFPAC-1 cells …… 19

Figure 7. PAUF knockdown effects on the cytotoxicity of 72 h inoculation with gemcitabine and 5FU in CFPAC-11 cells …… 21

Figure 8. PAUF knockdown effects on the mRNA expression of drug transporters and enzymes…… 22

LIST OF TABLES

Table 1. The sequences of PCR primer for the expression of known pancreatic CSCs related molecules ……7

<ABSTRACT>

PAUF is overexpressed in pancreatic cancer stem cells and confers chemoresistance.

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive malignancies. Similar to other tumors, pancreatic cancer stem cells (CSCs) have also been identified, and play an important role in development, maintenance, and metastasis of PDAC. Novel secretory protein of pancreatic adenocarcinoma up-regulated factor (PAUF) has been shown to contribute to cancer progression and metastasis. Because the clinical relationship between PAUF and pancreatic CSCs is largely unknown, we investigated the association between the functional role of PAUF and pancreatic CSCs. CFPAC-1 cancer spheres were cultured under nonadherent conditions with serum free media. PDAC spheres showed an elevated expression of PAUF protein and pluripotent stemness genes

(Oct4, Nanog, Stat3, and Sox2). The mRNA expression of PAUF was also upregulated in $CD44^{+}CD24^{+}ESA^{+}$ pancreatic CSCs. Sphere formation and soft agar assays showed diminished number of spheres and colonies by PAUF knockdown (shPAUF) in CFPAC-1 cells. Expression of stemness genes and CSC surface markers (CD133, c-MET and ALDH1) were also lower in shPAUF CFPAC-1 cells than in normal control cells. MTT assay revealed that PAUF silent (siPAUF) CFPAC-1 cells had lower mRNA expression of multidrug resistant protein 5 (MRP5) and ribonucleotide reductase M2 (RRM2) and were more vulnerable to gemcitabine and 5-FU than negative control cells ($p<0.05$). In conclusion, PAUF is a novel human pancreatic CSC-associated protein that may confer chemoresistance by modulating MRP5 and RRM2. Thus, PAUF may constitute a therapeutic target for overcoming drug-resistant pancreatic CSCs.

Key words: cancer stem cells, PAUF, pancreatic cancer, MRP5, RRM2

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I. INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive malignancies and is the fourth leading cause of cancer-related death in the western world.[1,2] The difficulty in early detection of PDAC and its resistance to conventional treatment options contribute to its dismal prognosis, as indicated by an overall 5-year survival rate of less than 10%.[1,2] Many researchers have attempted to elucidate the pathogenesis and carcinogenesis of PDAC. However, the molecular basis for the aggressive nature of PDAC is incompletely understood, and the overall survival of patients has not improved significantly despite advances in treatment modalities and introduction of novel targeted therapies.

The clinical outcomes of PDAC may be poor because conventional therapies are directed at tumor cells that have limited tumorigenic potential instead of

targeting the pancreatic cancer stem cells (CSCs) that have been identified in human PDAC.[3] Pancreatic CSCs are identified by a variety of biomarkers, and transplantation assays using immune-deficient mice show that pancreatic CSCs self-renew and propagate the parental tumor. Li et al. has identified populations of CSCs in PDAC that express the cell surface markers $CD44^+CD24^+ESA^+$ or c-Met.[3,4] Apart from these markers, $CD133^+$ and ALDH1 are regarded as other markers of CSCs in PDAC.[5-7] These CSCs are also characterized by their chemoresistance, as $CD133^+$ pancreatic cancer cells have greater drug resistance to gemcitabine,[6] and c-MET inhibitors have been shown to enhance antitumor effects in combination with gemcitabine.[3]

Genome-wide analyses have uncovered pancreatic adenocarcinoma up-regulated factor (PAUF), a novel secretory protein associated with pancreatic cancer.[8] Although the mechanism through which PAUF contributes to cancer progression is not clearly established, previous reports suggest it plays critical roles in PDAC progression and metastasis processes, including cell proliferation and modulation of adhesion, migration, and invasion. [9-15]

No studies have investigated the association between PAUF and CSCs and how it may contribute to drug resistance. Therefore, the purpose of the present study was to investigate the relationship between PAUF function and pancreatic CSCs. Our experiments provided evidence of PAUF expression in pancreatic CSCs and suggested that PAUF contributes to multi-drug resistance in the pancreatic CSCs

II. MATERIALS AND METHODS

1. Clinical samples and cell lines

Pancreatic cancer samples were obtained from surgical specimens at Myongji Hospital, Kwandong University of College of Medicine. Nine samples were obtained from pancreatic cancer resections, five from chronic pancreatitis resections, and eight from normal pancreas tissues obtained from benign pancreatectomy. The Ethics Committee for the Clinical Research of the Institutional Review Board of Myongji Hospital approved this study protocol.

Eight PDAC cell lines (AsPC-1, BxPC-3, Capan-1, Capan-2, CFPAC-1, HPAC, MiaPaca-2, and Panc-1) were obtained from ATCC(American Type Culture Collection). All cells were grown in the appropriate conditioned medium and maintained in an atmosphere of 5% CO_2/95% air at 37˚C. We primarily used CFPAC-1, which expresses high endogenous levels of PAUF, for our experiments.

2. Sphere culture

For formation of spheres, single cells were cultured for 7 days in Dulbecco's Modified Eagle Medium: Nutrient Mixture 12 (DMEM/F12) containing 0.5% fetal bovine serum (FBS) (Hyclone), 0.5% bovine serum albumin fraction V (Gibco), insulin-transferrin-selenium A (Gibco), 10 ng/ml human epidermal growth factor (hEGF; R&D systems, Wiesbaden-Nordenstadt, Germany), 10

ng/ml human fibroblast growth factor (hFGF; R&D), and 10 ng/ml human leukemia inhibitory factor (hLIF; R&D) at a density of 1×10^3 cells/ml in an ultralow attachment plate (Corning, Corning, NY, USA). Growth factors were added every 3 days. For preparation of secretory proteins, culture medium was changed to serum-free medium in the post sphere culture after 5 days and cultured for an additional 2 days.

3. Semi-quantitative PCR (reverse transcription PCR)

The total RNA from cancer cells was extracted using a RNA easy extraction kit (Qiagen) according to the manufacturer's instructions. To quantify the relative expression of the genes, we used β-actin primer as a control. The PCR primers used were PAUF sense, 5'-CACCTGGGCAGGGAAGATGTA-3'; PAUF antisense, 5'-GCTCAGTGGTCGGCTCCTCT-3'; β-actin sense, 5'-GGCATCCTCACCCTGAAGTA –3'; and β-actin antisense, 5'-GGGGTGTTGAAGGTCTCAAA-3'. The PCR primer sequences for the expression of genes related to pancreatic CSCs are described in Table 1.

Table 1. The sequences of PCR primer for the expression of known pancreatic CSCs related molecules

Name	Sequence
ß-catenin (sense)	GTATGAGTGGGAACAGGGATTT
ß-catenin (antisense)	CCTGGTCCTCGTCATTTAGC
SOX2 (sense)	CACAACTCGGAGATCAGCAA
SOX2 (antisense)	GTTCATGTGCGCGTAACTGT
LEF1 (sense)	CGAAGAGGAAGGCGATTTAG
LEF1 (antisense)	GGATGGGTGGAGAAAGAGAT
WNT4 (sense)	TTGAGGAGTGCCAGTACCAG
WNT4 (antisense)	CGTAGGCGATGTTGTCAGAG
NANOG (sense)	ACTGTCTCTCCTCTTCCTTCCT
NANOG (antisense)	AGAGTAAAGGCTGGGGTAGGTA
IHH (sense)	CCTGAACTCGCTGGCTATCT
IHH (antisense)	AATACACCCAGTCAAAGCCG
JAGGED1 (sense)	CTCAATTACTGTGGGACTCA
JAGGED1 (antisense)	GAACACTCACACTCAAAGCC
GLI-1 (sense)	CCTACCAGAGTCCCAAGTTT
GLI-1 (antisense)	AGAGTCCAGGGGGTTACATA
NOTCH3 (sense)	ATGGTGGGAACTAAACACAGCT
NOTCH3 (antisense)	ATGACCCTGGAGGAAGCACA
PTCH (sense)	CTCGGATTGGTGACCATAAG
PTCH (antisense)	GCSGTGGTGAGAGAAAAGGA
DVL2 (sense)	GGTTGGGGAGACGAAGGTGATT
DVL2 (antisense)	ATCTGAGGACACCAGCCAGGATAC
HES1 (sense)	GTGCTGTCTGGATGCGGAGT
HES1 (antisense)	ATTCCTGCTCTCGCCTTCG
OCT4 (sense)	AAG TGG GTG GAG GAA GCT
OCT4 (antisense)	CGA GGA GTA CAG TGC AGT
STAT3 (sense)	GTCTGGCTGGACAATATCAT
STAT3 (antisense)	TTGGGAATGTCAGGATAGAG
CD44 (sense)	CACCATTTCAACCACACCAC
CD44 (antisense)	GGTGTTGTCCTTCCTTGCAT
CD133 (sense)	GCTCAGACTGGTAAATCCCC
CD133 (antisense)	GACTCGTTGCTGGTGAATTG
c-MET (sense)	CAA TGT GAG ATG TCT CCA GC
c-MET (antisense)	CCT TGT AGA TTG CAG GCA GA
ALDH1A1 (sense)	AGCAGGAGTGTTTACCAAAGA
ALDH1A1 (antisense)	CCCAGTTCTCTTCCATTTCCAG
ABCG2 (sense)	CAC TGA TCC TTC CAT CTT GT
ABCG2 (antisense)	TAT GAG TGG CTT ATC CTG CT
VIMENTIN (sense)	GAG AAC TTT GCC GTT GAA GC

VIMENTIN (antisense)	GCT TCC TGT AGG TGG CAA TC
N-CADHERIN (sense)	ACAGTGGCCACCTACAAAGG
N-CADHERIN (antisense)	CCGAGATGGGGTTGATAATG
E-CADHERIN (sense)	TGC CCA GAA AAT GAA AAA GG
E-CADHERIN (antisense)	GTG TAT GTG GCA ATG CGT TC
SNAIL (sense)	AAG CTT CCA TGG CGC GCT CTT TCC TCG TCA GGA AGC CC
SNAIL (antisense)	GGA TCC TCA GCG GGG ACA TCC TGA GCA GCC GGA CTC TTG
SLUG (sense)	AGC GAA CTG GAC ACA CAT ACA
SLUG (antisense)	CTG AGC CAC TGT GGT CCT T

4. Establishment of stable PAUF knockdown cell line using shRNA

The shRNA-expressing plasmid targeting human PAUF and negative control plasmid were purchased from SABiosciences. The human PAUF shRNA sequence was 5'-ACACCAGCAAGGACCGCTATT -3' and the control shRNA sequence 5'-GGAATCTCATTCGATGCATAC -3'. For shRNA transfection, CFPAC-1 cells were plated into 6-well plates at a density of 5×10^4 cells/well the day before transfection. Transfection was performed using Lipofectamine2000 reagent according to manufacturer's instructions, and stable knockdown clones were selected using neomycin

5. Transient transfection of small interfering RNA (siRNA) targeted for PAUF

The two sets of 25-nucleotide stealth RNAi-targeting PAUF were synthesized customarily by Invitrogen. Stealth RNAi duplexes, which have a similar GC content to that of the duplex siRNA obtained from Invitrogen, were used as

negative controls. Stealth RNAi-targeting PAUF were transfected into CFPAC-1 cells using Lipectamine™ RNAi Max transfection agent (Invitrogen) according to the manufacturer's protocol. Cells were harvested 72 h post-transfection and subjected to total RNA extraction and a migration and invasion assay. Secreted protein was prepared from culture medium 48 h post-transfection.

6. MTT assay

After incubation at 37˚C overnight, cells were treated with various concentrations of gemcitabine or fluorouracil (5-FU) in complete growth media and incubated for 72 h at 37˚C. A 3-(4,5-dimenthelthiazol-2-ly)-2,5 -diphenyltetrasolium bromide-based assay (absorbance 570 nm) was used to measure the number of metabolically active cells

7. Flow cytometry and cell sorting

Cultured cells were detached with accutase solution (Sigma-Aldrich, Inc., St. Louis, MO, USA) and washed in phosphate-buffered saline (PBS) containing 0.5% FBS. Single cells were stained for 20 min on ice in the dark, washed twice in PBS containing 0.5% FBS, and fixed in 2% paraformaldehyde. Flow cytometry analysis was performed using a FACSCalibur system (BS Biosciences, San Jose, CA), and cell sorting was performed using FACSAria II (BD Immunocytochemistry System, Franklin Lakes, NJ). Antibodies against

CD24 (anti-CD24-PE, BD), CD44 (anti-CD44-APC, BD), and ESA (anti-ESA-FITC, BD) were used. FITC-mouse IgG2b, κ isotype control (BD), rat IgG1 κ isotype control FITC (eBioscience), PE-mouse IgG2a, κ isotype control (BD), and APC-mouse IgG2b, κ isotype control (BD) were used as controls.

8. Protein extraction and western blot

Cells were prepared in lysis buffer containing 50 mM HEPES (pH 7.2), 150 mM NaCl, 25 mM beta-glycerophosphate, 25 mM NaF, 5 mM EGTA, 1 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate, 0.1 mM phenylmethanesulfonylfluoride (PMSF), and a protease inhibitor cocktail (Roche Diagnostics). For secretory protein preparation, culture medium was centrifuged and the cellular components and debris were discarded. Culture medium was concentrated by the addition of ice-cold acetone, and the precipitated protein was resuspended with lysis buffer. Proteins were separated on SDS-PAGE and transferred to 0.45-μm Immobilon P-transfer membrane (Millipore). Membrane was blocked in 5% (w/v) non-fat milk and probed with the following primary antibodies: anti-human PAUF antibody (2009 Cancer Science), Sox2 (Cell Signaling Technology), Oct4 (Cell Signaling Technology), Nanog (Cell Signaling Technology), aldehyde dehydrogenase (ALDH) (BD), Cdk6 (Santa Cruz), cyclin D3 (Santa Cruz), Snail (Cell Signaling Technology), slug (Cell Signaling Technology), twist (Cell Signaling Technology) and

glyceraldehyde phosphate dehydrogenase (GAPDH) (Santa Cruz). Immunoreactive material was then visualized using SuperSignal West Pico Chemiluminescence Substrate[16] according to the manufacturer's instructions.

9. Immunohistochemistry

Paraformalin-fixed paraffin-embedded tissue sections (3-5-μm thickness) were deparaffinized in xylene, rehydrated with a graded ethanol series (100-95-90-80-70-50-30%), and washed with PBS. Endogenous peroxidase was blocked by immersing the slide in 0.3% (v/v) hydrogen peroxide in methanol for 10 min at room temperature. Microwave antigen retrieval was performed in citrate buffer (0.01 M, pH 6.0). The sections were blocked by soaking in 10% (v/v) normal donkey serum for 1 h and incubated with anti-human PAUF primary antibody (1:600) overnight at 4˚C. The sections were incubated with EnVision/HPR, Rabbit/Mouse (DakoCytomation, CA, USA) and diaminobenzidine (DAB+) chromogen. The sections were counterstained with hematoxylin (Sigma-Aldrich, Inc., St. Louis, MO, USA), dehydrated, and mounted.

10. Soft agar assay

A suspension of 500 single cells containing 0.3% agar medium was overlaid on 0.6% agar medium in 24-well plates. Each well was covered with complete medium, and the plates were incubated for 4 weeks. Colonies were stained with

crystal violet and counted. Experiments were done in triplicate.

11. Statistical analysis

Mann-Whitney U test and independent *t*-test were used to compare cell survival between siPAUF and control groups. Statistical calculations were performed using SPSS (version 12.0 for Windows; SPSS, Inc., Chicago, IL, USA). Values of $p < 0.05$ were considered statistically significant.

III. RESULTS

1. Expression of PAUF protein in human pancreatic cancer

Immunohistochemistry was performed to investigate the relationship between PAUF expression and clinicopathological features of pancreatic cancer. PAUF was aberrantly expressed in the all of nine pancreatic cancer specimens, as shown by the high-intensity pattern of cytoplasmic staining. This pattern was not observed in the five chronic pancreatitis or eight normal pancreas specimens (Fig 1).

(A) **(B)**

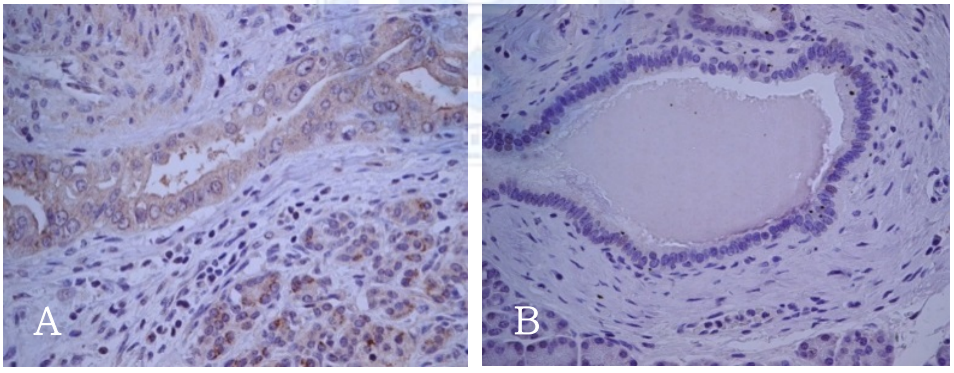


Figure 1. Validation of PAUF expression in pancreatic tissues. (A) pancreatic cancer and (B) normal pancreatic tissue were stained with anti-PAUF antibodies. Pancreatic cancer showed positive cytoplasmic staining of PAUF, whereas normal pancreatic tissue showed negative staining of PAUF (×400).

2. Pancreatic cancer spheres cultured from human pancreatic cancer cell lines

We used sphere culture to identify CSCs. This *in vitro* method involved culturing candidate pancreatic CSCs with serum-free media containing only EGF and bFGF under nonadherent conditions, and the resulting spheres indicated self-renewal consistent with a CSC phenotype. In this study, PDAC spheres cultured from the CFPAC-1 cell line showed upregulated expression of the pluripotent stemness genes Oct4, Nanog, Stat3, and Sox2 relative to adherent CFPAC-1 cells (Fig 2).

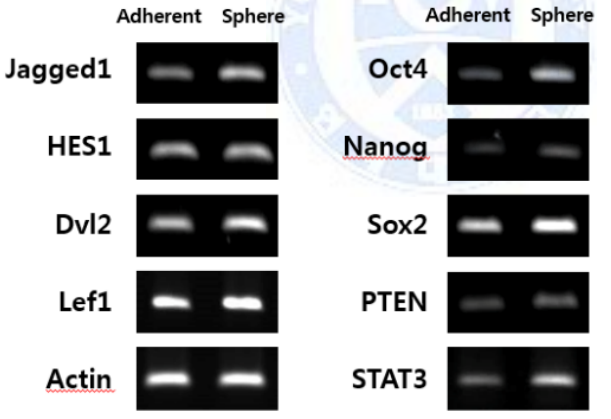


Figure 2. Expression of pluripotent stemness genes in pancreatic cancer spheres formed from CFPAC-1 cells. RT-PCR showed that the overexpression of Oct4, Nanog, Stat3, and Sox2 genes in CFPAC-1 sphere compare to adherent cells.

3. PAUF overexpression in pancreatic CSCs

Based on previous reports that identified the cell surface markers $CD44^+CD24^+ESA^+$ in a CSC population of human PDAC, we sorted the CFPAC-1 cell line into $CD44^+CD24^+ESA^+$ PDAC cells using fluorescence-activated cell sorting (FACS) and determined PAUF gene expression using RT-PCR. To confirm the relationship between PAUF and CSCs, we measured the mRNA expression of PAUF in $CD44^+CD24^+ESA^+$ pancreatic CSCs and the protein content of PAUF in PDAC spheres using western blots. PAUF protein content was higher in CFPAC-1 spheres than in adherent cells. Additionally, PAUF mRNA and protein content was higher in $CD44^+CD24^+ESA^+$ CFPAC-1 than in $CD44^-CD24^-ESA^-$ CFPAC-1 cells (Fig 3).

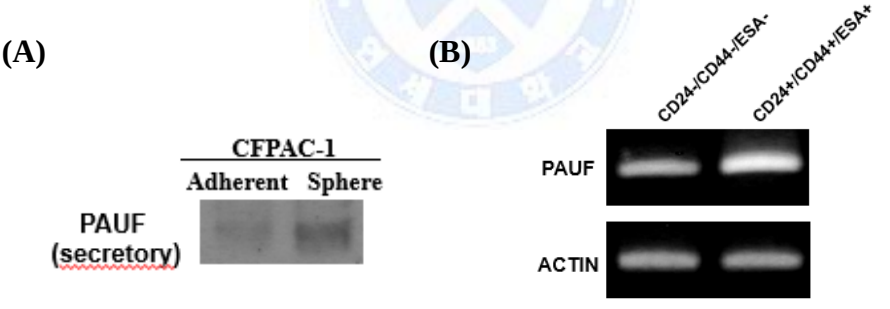


Figure 3. PAUF overexpression in pancreatic cancer stem cells. (A) Up-regulation of secretory PAUF was determined by western blot in pancreatic cancer spheres formed from pancreatic cancer cells. (B) RT-PCR demonstrated that the mRNA expression of PAUF was higher in $CD24^+/CD44^+/ESA^+$ cells than in $CD24^-/CD44^-/ESA^-$ pancreatic cancer cells.

4. PAUF knockdown effect on pancreatic CSCs

To further access the specific role of PAUF in pancreatic CSCs function, we knocked down PAUF expression using shRNA targeting regions of PAUF. The shPAUF CFPAC-1 cells showed reduced cell proliferation, migration, and wound healing ability. The effect of PAUF knockdown, determined using sphere formation and soft agar assay, showed that negative control CFPAC-1 formed spheres and that shPAUF CFPAC-1 cells had markedly diminished formation of spheres. Furthermore, the soft agar assay showed that the number of colonies formed was also lower in the shPAUF CFPAC-1 cells (Fig. 4).

The mRNA and protein expression of pluripotent stemness genes in shPAUF CFPAC-1 was measured using RT-PCR and western blot, respectively. The expression of stemness-related genes (including Oct4, Nanog, and Sox2), CSC surface markers (such as CD133, c-Met, and ALDH1), and the epithelial-mesenchymal transition marker, β-catenin were lower in the shPAUF CFPAC-1 than in the normal CFPAC-1 cells. Protein expression of PAUF, Sox2, ALDH, cyclin D3 and twist were lower in shPAUF CFPAC-1 than in control cells (Fig 5).

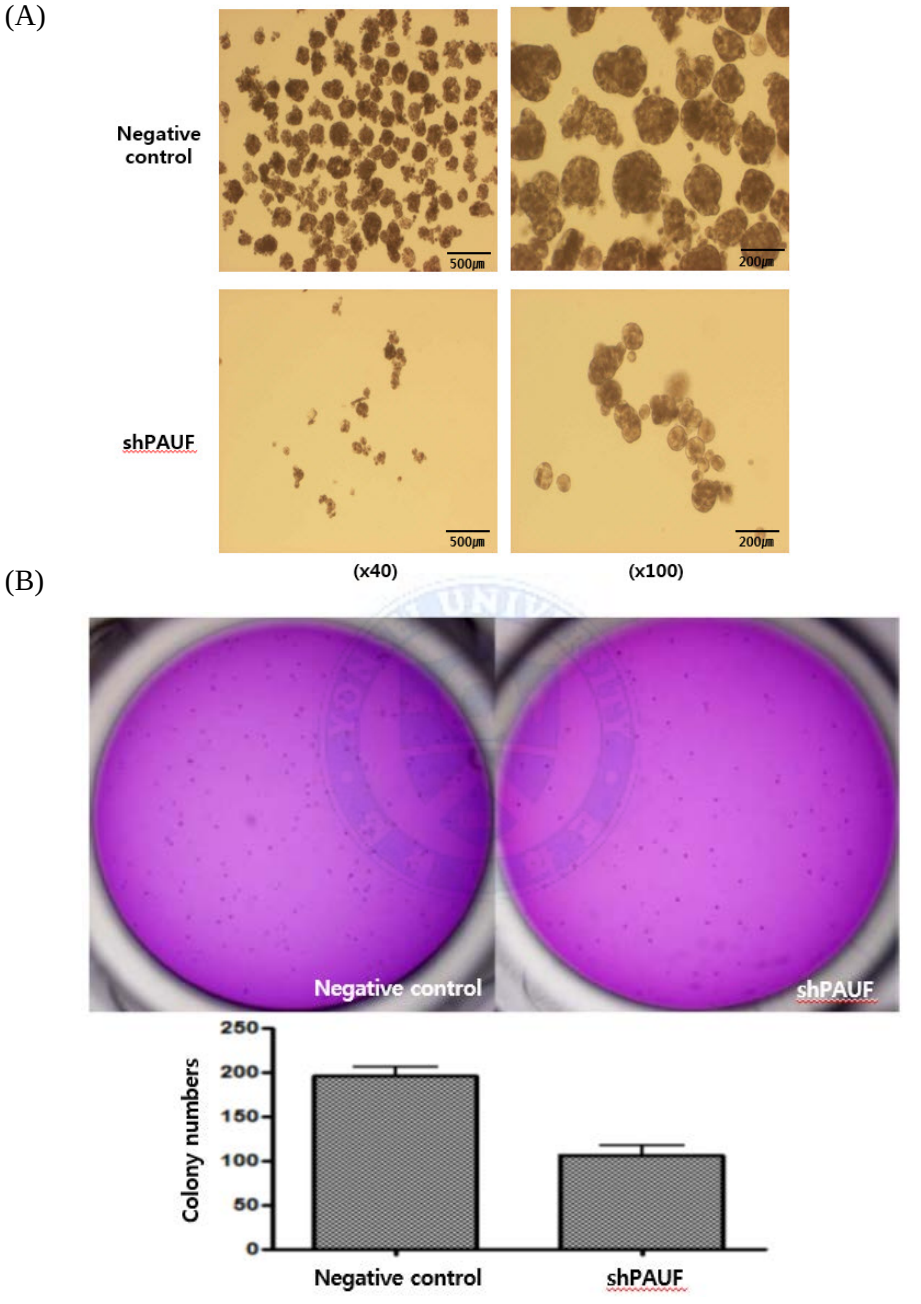


Figure 4. Effect of PAUF knockdown on sphere- and colony-forming ability.

(A) Sphere formation assays were performed in negative control and shPAUF

CFPAC-1 cells. Knockdown of PAUF expression reduced sphere-forming ability. (B) Soft agar assays demonstrated decreased number of colonies formed in shPAUF CFPAC-1 cells than in negative control cells.

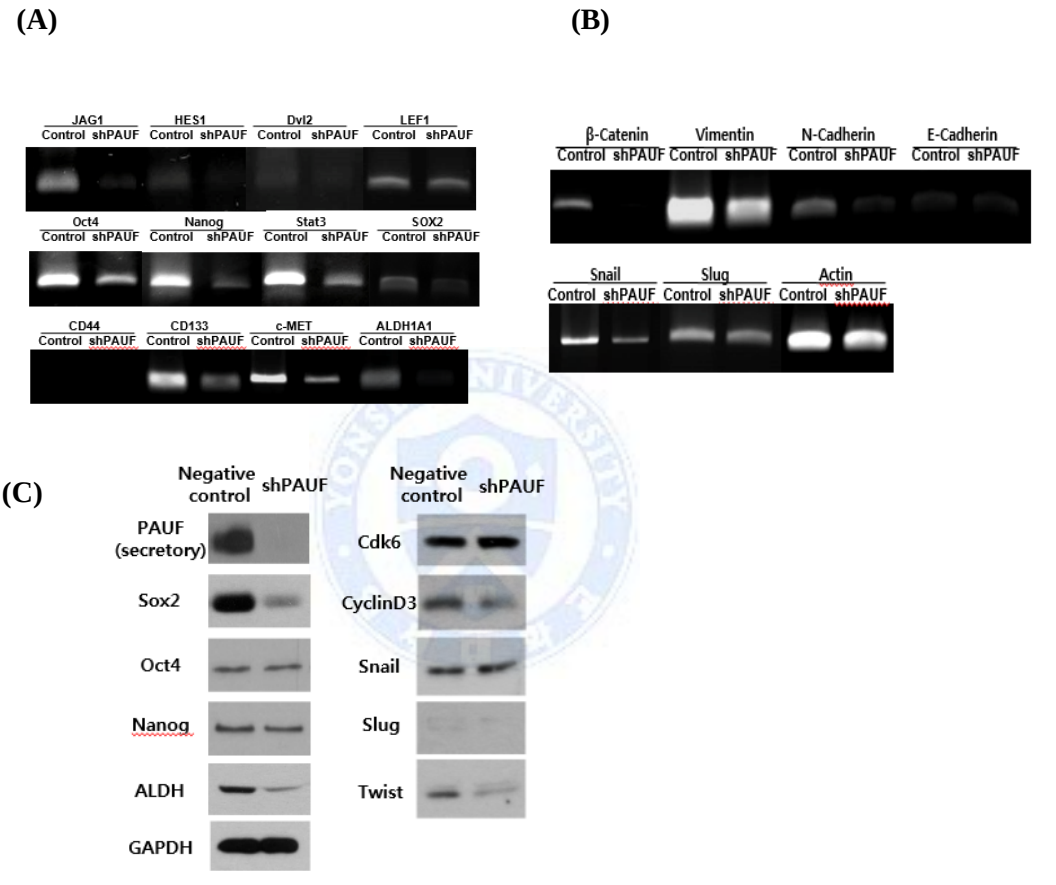


Figure 5. Differential gene expression in PAUF knockdown CFPAC-1 cells. (A) Expression of pluripotent stemness genes in shPAUF CFPAC-1 cells was determined by RT-PCR. (B) Expression of cancer stem cell and epithelial-mesenchymal transition markers were determined by RT-PCR. (C) Cancer stem cell-related proteins were validated by western blot.

We used FACS to determine the expression of pancreatic CSC-related surface markers CD24/CD44/ESA in siPAUF CFPAC-1 cells. The siPAUF CFPAC-1 cells had a relatively smaller proportion of $CD44^{+}CD24^{+}ESA^{+}$ CFPAC-1 CSCs than the cells with normal PAUF expression. (Fig 6)

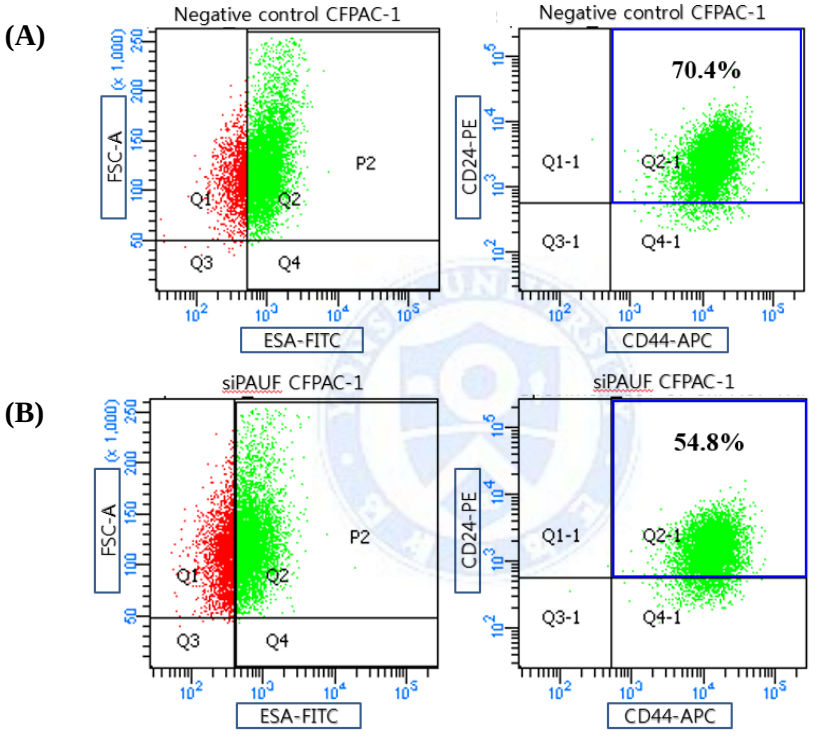
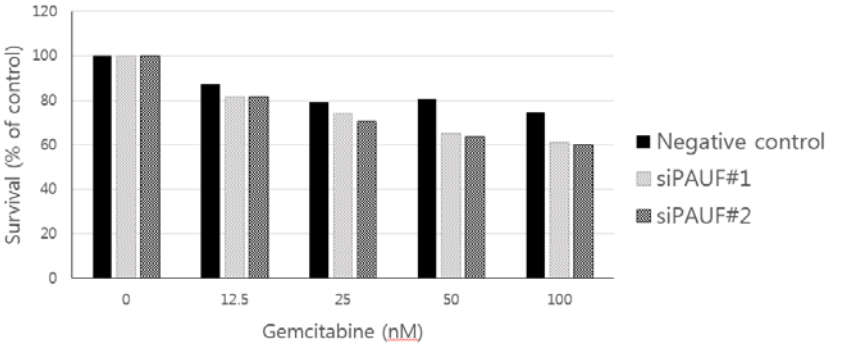


Figure 6. PAUF knockdown effects on the expression of pancreatic cancer stem cell markers (CD24/CD44/ESA) in CFPAC-1 cells. FACS analysis was used to determine the expression of pancreatic cancer stem cell markers (CD24/CD44/ESA) in negative control CFPAC-1 (A) and siPAUF CFPAC-1 cells (B).

5. Effects of PAUF on chemoresistance of pancreatic cancer cell lines

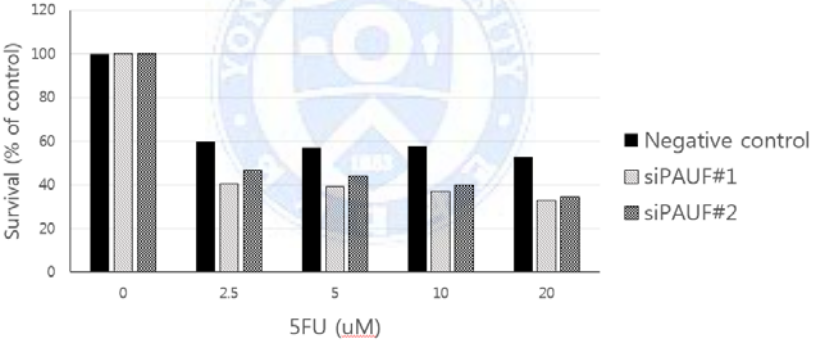
Past studies have suggested that pancreatic CSCs are more resistant to anti-cancer chemotherapy agents such as gemcitabine and 5-FU. To determine the role of PAUF in chemoresistance, we performed cell survival assays to validate cytotoxic effects of 5-FU or gemcitabine against PAUF-associated pancreatic cancer. Two transient siPAUF RNAs were used for this experiment. Both siPAUF CFPAC-1 cell lines were more vulnerable to the two cytotoxic drugs than the control CFPAC-1. ($p<0.05$, Fig. 7) Furthermore, mRNA expression of multidrug resistant protein 5 (MRP5) and ribonucleotide reductase M2 (RRM2) was lower in siPAUF CFPAC-1 cells than in negative control cells (Fig 8).

(A)



Gemcitabine (nM)	Negative control	siPAUF#1	siPAUF#2	*p*-value
0	100	100	100	
12.5	87.1	84.6	81.7	
25	79.4	74.1	70.4	
50	80.9	65.2	63.8	
100	74.6	61.0	60.0	<0.01

(B)



5FU (nM)	Negative control	siPAUF#1	siPAUF#2	*p*-value
0	100	100	100	
25	59.8	40.7	46.7	
5	56.9	39.2	44.0	
10	57.9	36.9	39.8	
20	52.9	33.0	34.5	<0.01

Figure 7. PAUF knockdown effects on the cytotoxicity of 72 h inoculation with gemcitabine and 5FU in CFPAC-1 cells. MTT assay showed significantly increased antitumor effect of gemcitabine (A) and 5FU (B) in PAUF-silenced CFPAC-1 cells compared to negative control.

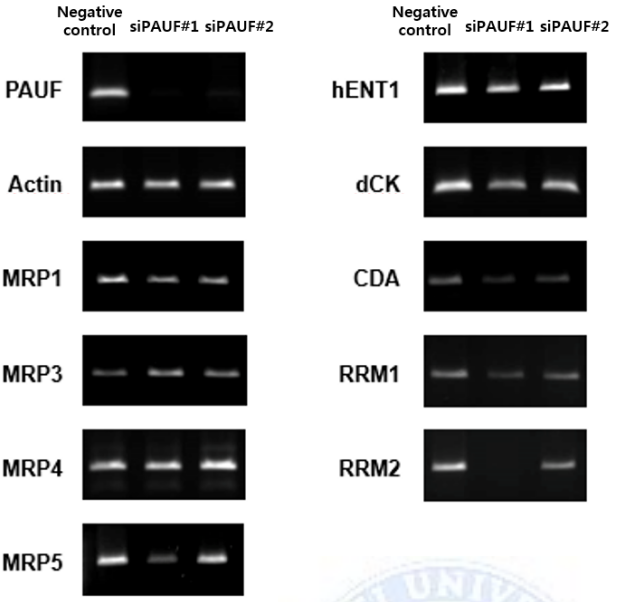


Figure 8. PAUF knockdown effects on the mRNA expression of drug transporters and enzymes. RT-PCR demonstrated mRNA expression of MRP5 and RRM2 was lower in the siPAUF CFPAC-1 cells than in the negative control.

IV. DISCUSSION

Recent studies have demonstrated the presence of pancreatic CSCs, which are capable of self-renewal and production of differentiated progeny, in PDAC. Although the isolation and culturing of pancreatic CSCs remains difficult, the role of CSCs in development, maintenance, and metastasis of PDAC is increasingly recognized. In this study, we cultured the pancreatic cancer cell line CFPAC-1 in serum-free media and obtained pancreatic cancer sphere cells, which had self-renewal capabilities. Relative to the adherent cells, PDAC spheres showed increased mRNA and protein expression of PAUF and the pluripotent stemness genes Oct4, Nanog, Sox2 and STAT3. Additionally, $CD44^{+}CD24^{+}ESA^{+}$ pancreatic CSCs had increased mRNA expression of PAUF.

We recently identified PAUF, (also known as a paralog of ZG16B), a novel 27-kDa secretory protein highly expressed in human PDAC. [8] As an autocrine factor, PAUF is involved in altered migration, invasion, proliferation, and metastasis.[9,11,17] PAUF also acts as a paracrine factor by causing stromal changes in the tumor microenvironment that promotes tumor evasion of the immune surveillance system.[12] PAUF also modulates permeability of the endothelial cells and vasculature to promote tumor angiogenesis.[18] Although only a few preclinical studies have investigated anti-PAUF monoclonal antibodies, PAUF-specific RNA aptamers (P12FR2), and adenoviral PAUF-targeting trans-splicing ribozymes (TSR) for PDAC, PAUF-targeted therapies may be an alternative approach for cancer treatment.[10,14,15] The

above-mentioned studies suggest functional roles of PAUF in pancreatic cancer; however, little is known about the clinical relationship between PAUF and pancreatic CSCs. Therefore, we show the association between PAUF expression and characteristics of CSCs to determine the contribution of PAUF activity to chemoresistance in pancreatic CSCs,

Of clinical importance, recent research suggests CSCs are resistant to conventional chemotherapy and radiation.[4] Enhanced expression of ATP-binding cassette (ABC) family of transporters has been shown to be characteristic of side population cells with CSC features. These side population cells are more resistant to chemotherapeutic agents and may play a key role in tumor progression, recurrence, and metastasis.[19,20] To identify the relationship between chemoresistance and the functional role of PAUF in this study, we conducted cytotoxic studies with gemcitabine and 5-FU in siPAUF CFPAC-1 and negative control cells. Our study showed that siPAUF CFPAC-1 cells were significantly more vulnerable to both cytotoxic drugs. Because the chemoresistant mechanisms can occur through intracellular enzymes, uptake, and/or export drug transporters, we measured the mRNA expression of genes involved in drug metabolism (such as dCK, CDA, RRM1, and RRM2) and transporters (including MRP3, MRP4, MRP5, and hENT1). In the present study, the siPAUF CFPAC-1 cells had lower mRNA expression of MRP5 and RRM2. MRP5 (also known as ABCC5) is a member of the multidrug resistance-associated protein subfamily of ABC transporters and confers

resistance against chemotherapeutic drugs such as etoposide, 5-FU, and gemcitabine.[21-23] RRM2 is the catalytic subunit of ribonucleotide reductase, a dimeric enzyme that supplies deoxynucleotides essential for DNA replication and cell growth. The overexpression of RRM2 has been associated with gemcitabine resistance and increased cellular invasiveness *in vitro*,[24 25] and high expression of RRM2 in pancreatic tumors is associated with reduced overall survival after resection.[26]

Taken together, our study indicated that PAUF signaling is required for the survival of pancreatic CSCs. In addition, PAUF may contribute to drug resistance through mechanisms involving MRP5 and RRM2, thus making it a novel target for treatment of PDAC. However, our study had some limitations. We used pancreatic cancer cell lines rather than patient-derived pancreatic cancer cells for many of our experiments and only investigated the effect of PAUF knockdown in PDAC cancer cell lines rather than in pancreatic CSCs derived from human tissues. In addition, we did not perform the experimental study using *in vivo* models of human PDAC, which are required to prove a causal relationship between PAUF and target proteins such as MRP5 and RRM2. Thus, the possible connection between PAUF, drug transporters, and cell signaling pathways should be investigated in future studies.

V. CONCLUSION

In conclusion, we showed that PAUF is a novel protein in human pancreatic CSCs that may contribute to chemoresistance by modulating MRP5 and RRM2. Thus, PAUF may constitute a therapeutic target to reduce the drug resistance of pancreatic CSCs.

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항암제 내성 증가와 연관된 PAUF 단백의 췌장암줄기세포 내 과발현

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조재희

췌장암은 예후가 불량한 암으로 서구에서는 암 연관 사망의 4번째 원인이다. 췌장암줄기세포는 췌장암의 발생, 유지, 전이의 중요한 역할을 하고 있는 것으로 알려져 있고, 최근 발견된 PAUF (pancreatic adenocarcinoma up-regulated factor) 단백은 암의 진행과 전이와 연관됨이 보고되었다. 이에 저자 등은 췌장암줄기세포와 PAUF의 연관성을 확인하고 작용 기전을 연구하고자 하였다.

CFPAC-1 췌장암 세포주를 serum free media 의 nonadherent 환경에서 췌장암 sphere로 배양하였고, Western blot과 RT-PCR 실험을 진행하여 PAUF 단백과 Oct4, Nanog, Stat3, and Sox2 등의 줄기세포 연관 mRNA의 과발현을 확인하였다. 또한 CFPAC-1 췌장암세포주를 FACS 분석을 통해 췌장암줄기세포로 생각되는 $CD44^{+}CD24^{+}ESA^{+}$ CFPAC-1로 분리하고, PAUF mRNA의 과발현을 관찰하였다.

PAUF의 발현 억제와 암줄기세포의 연관성을 확인하기 위해 다음과 같은 실험이 추가로 진행하였다. PAUF knockdown (shPAUF) CFPAC-1 췌장암세포주에 대한 sphere formation과 soft agar assay 실험에서 shPAUF CFPAC-1은 sphere 및 colony 형성이 모두 감소하였고, 줄기세포 연관 유전자와 암줄기세포 표면 표시 인자(CD133, c-MET and ALDH1)의 mRNA가 감소되었다. PAUF silent (siPAUF) CFPAC-1 세포주를 이용한 MTT 분석에서 Gemcitabine과 5-FU 항암제 감수성은 PAUF를 억제하는 경우 증가하였으며, siPAUF CFPAC-1에서 MRP5(multidrug resistant protein 5)와 RRM2(ribonucleotide reductase M2) mRNA의 발현 저하가 확인되었다.

결론 하자면, PAUF 는 췌장암 줄기세포와 연관 단백질로, MRP5와 RRM2의 발현을 조절하여 항암제 내성과 연관성이 있다. 그러므로, 추후 PAUF는 췌장암줄기세포의 항암제내성 극복을 위한 표적 물질로 사용이 기대된다.

핵심 되는 말: 암줄기세포, PAUF, 췌장암, MRP5, RRM2

PUBLICATION LIST

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ACRONYMS LIST

Pancreatic ductal adenocarcinoma: PDAC

Cancer stem cells: CSCs

Pancreatic adenocarcinoma up-regulated factor: PAUF

Octamer-binding transcription factor 4: Oct4

Signal transducer and activator of transcription 3: Stat3

Sex determining region Y-box 2: Sox2

Epithelial-specific antigen: ESA

Aldehyde dehydrogenase: ALDH

Phosphatase and tensin homolog: PTEN

Lymphoid enhancer-binding factor 1: Lef1

Dishevelled segment polarity protein 2: Dvl2

Multidrug resistant protein: MRP

Ribonucleotide reductase M: RRM

American Type Culture Collection: ATCC

Human epidermal growth factor: hEGF;

Human fibroblast growth factor: hFGF

Human leukemia inhibitory factor: hLIF;

Fluorescence-activated cell sorting: FACS

Cyclin-dependent kinase 6: CDK6

Deoxycytidine kinase: dCK

Equilibrative nucleoside transporter 1: ENT1